Cell-autonomous roles of the *ecdysoneless* gene in *Drosophila* development and oogenesis

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Summary

Steroid signaling underlies developmental processes in animals. Mutations that impair steroidogenesis in the fruit fly *Drosophila melanogaster* provide tools to dissect steroid hormone action genetically. The widely used temperature-sensitive mutation *ecdysoneless[1]* (*ecd[1]*) disrupts production of the steroid hormone ecdysone, and causes developmental and reproductive defects. These defects cannot be satisfactorily interpreted without analysis of the *ecd* gene. Here, we show that *ecd* encodes an as yet functionally undescribed protein that is conserved throughout eukaryotes. The *ecd[1]* conditional allele contains an amino acid substitution, whereas three non-conditional larval lethal mutations result in truncated Ecd proteins. Consistent with its role in steroid synthesis, Ecd is expressed in the ecdysone-producing larval ring gland. However, development of *ecd*-null early larval lethal mutants cannot be advanced by Ecd expression targeted to the ring gland or by hormone feeding. Cell-autonomous *ecd* function, suggested by these experiments, is evidenced by the inability of *ecd*$^{-}$ clones to survive within developing imaginal discs. Ecd is also expressed in the ovary, and is required in both the follicle cells and the germline for oocyte development. These defects, induced by the loss of *ecd*, provide the first direct evidence for a cell-autonomous function of this evolutionarily conserved protein.

Key words: Steroid hormone, Ecdysone, *ecdysoneless*, Imaginal disc, Oogenesis, *Drosophila*

Introduction

Steroid hormones play crucial roles in development and reproduction of insects, including the fruit fly *Drosophila melanogaster*. The insect steroid ecdysone (E), and primarily its active derivative 20-hydroxyecdysone (20E), is responsible for coordination of embryogenesis, larval molting and metamorphosis, the latter involving differentiation of adult structures from precursor imaginal discs (Riddiford, 1993). We will hereafter use the generic name ecdysone to refer to the *Drosophila* steroid hormone. Blood-circulating ecdysone induces tissue-specific and temporally restricted proliferation, differentiation and programmed cell death. Numerous studies, directed towards understanding how the ubiquitous hormone governs these diverse cellular responses, culminated in detailed dissection of the regulatory cascade downstream of the ecdysone signal (Thummel, 1996).

The major and best-studied source of ecdysone in insect larvae is the prothoracic gland, which in *Drosophila* consists of the lateral lobes of the ring gland (Dai and Gilbert, 1991). After this part of the ring gland degenerates during metamorphosis, adult ovaries contribute to the whole body steroid titer in *Drosophila* (Garen et al., 1977; Bownes et al., 1984; Bownes, 1989; Warren et al., 1996). The main role of ecdysone in adult females is to regulate vitellogenesis (Hagedorn, 1985; Bownes et al., 1996). In addition, ecdysone has been implicated in egg chamber maturation during mid-oogenesis (Buszczak et al., 1999). Inactive ecdysone conjugates are maternally deposited to eggs and are mobilized during mid-embryogenesis by the amnioserosa (Bownes et al., 1988; Kozlova and Thummel, 2003).

Recently, several *Drosophila* genes involved in ecdysone biosynthesis have been cloned. One is *dare*, a homolog of the human adrenodoxin reductase that is necessary for the reduction of mitochondrial cytochrome P450 (Cyp) enzymes (Freeman et al., 1999). Two other genes, *disembodied* (*dib*) and *shadow* (*sad*), encode Cyp C_{22}- and C_2-hydroxylases, respectively, which are responsible for the final two hydroxylation steps of ecdysone synthesis (Chavez et al., 2000; Warren et al., 2002). Ecdysone is the final product of the ring gland, which is secreted to the hemolymph and converted to 20E in peripheral tissues. The Cyp C_{20}-hydroxylase responsible for this conversion is encoded by *shade* (*shd*) (Petryk et al., 2003). The *dare*, *dib* and *sad* genes are all expressed in the larval lateral ring gland and in adult ovaries, and their loss-of-function phenotypes can be fully explained as a consequence of ecdysone deficiency. Thus far, only one steroidogenic factor that is not itself an enzyme, *without children* (*woc*), has been identified (Wismar et al., 2000; Warren et al., 2001). This gene encodes a zinc finger transcription factor that probably activates expression of the cholesterol 7,8-dehydrogenase that executes the first step of

ecdysone biosynthesis. Mutations of *woc* affect a wide range of tissues, suggesting that its transcriptional function is not restricted to regulating expression of the steroidogenic enzyme. No other regulators of the steroidogenic pathway have been identified thus far.

Among steroid-deficient *Drosophila* mutations, *ecdysoneless*^{1} (*ecd*^{1}) is used to study ecdysone roles in development. The *ecd*^{1} mutation is a recessive, temperature-sensitive allele that reduces whole-body ecdysone titers and causes larval arrest at a restrictive temperature, 29°C (Garen et al., 1977). The effect of *ecd*^{1} on ecdysone production is autonomous, because cultured *ecd*^{1} mutant ring glands fail to produce ecdysone when upshifted to 29°C (Henrich et al., 1987; Dai et al., 1991; Warren et al., 1996). Ecdysone production is also interrupted in adult ovaries upshifted to the restrictive temperature (Garen et al., 1977; Redfern and Bownes, 1983; Warren et al., 1996). After several days at 29°C, oogenesis pauses at the onset of vitellogenesis; this phenotype can be reversed by lowering the temperature (Audit-Lamour and Busson, 1981). Transplantation experiments show that this effect of *ecd*^{1} is autonomous to the ovary (Garen et al., 1977).

Developmental events disrupted in *ecd*^{1} mutants include fat body protein synthesis (Lepesant et al., 1978), progression of the eye-forming morphogenetic furrow (Brennan et al., 1998), salivary gland glue secretion (Biyasheva et al., 2001) and motor neuron outgrowth (Li and Cooper, 2001). These defects have been interpreted as consequences of the mutationally induced ecdysone deficiency. However, Redfern and Bownes caution that a range of anomalies in *ecd*^{1} adults result from an autonomous *ecd* requirement for cell viability and therefore may not be attributable to ecdysone deficiency (Redfern and Bownes, 1983).

It is difficult to discern which of the phenotypes result from the *ecd*^{1} mutation directly, and which are the consequence of low ecdysone titer, without knowing the primary defect in the *ecdysoneless* gene, whose molecular identity remained elusive for over 25 years. We report here that the *ecd* locus encodes a protein whose orthologs in several other species, including humans, have not yet been functionally described. The original *ecd*^{1} mutation and three non-conditional lethal alleles have been mapped and assessed for their effects. We have localized the Ecd protein to both the steroidogenic and non-steroidogenic tissues, and have demonstrated its cell-autonomous roles in imaginal discs and ovaries.

Materials and methods

Drosophila strains

Flies were cultured on standard cornmeal medium at 25°C unless otherwise specified. The *ecd* mutations examined in this study included the temperature-sensitive *ecd*^{1} (Garen et al., 1977) and three non-conditional recessive lethals: EMS-induced alleles *ecd*^{2} (*ru ecd*^{2} *st e*) (Sliter et al., 1989) and *ecd*$^{l(3)23}$ (a gift of Dr I. Zhimulev), and a γ-ray-induced *ecd*^{g24} (*ve R ecd*^{g24}) (V.C.H., unpublished). Deficiencies *Df(3L)R+R2* and *Df(3L)Aprt201* were from previous irradiation screens (Sliter et al., 1989; Wang et al., 1994). The *mbf1*-null mutant line (Liu et al., 2003) was used for control in the analyses of mitotic mutant clones.

Genetic mapping and sequence analysis of *ecd*

Deficiencies *Df(3L)R+R2*, in the 62B-D chromosomal region that deletes the *ecd* locus (Sliter et al., 1989), and *Df(3L)Aprt201*, which complements the non-conditional *ecd* alleles, were used to delimit the *ecd* interval by a series of PCR reactions. These were performed on embryos homozygous for either *Df(3L)Aprt201* or *Df(3L)R+R2* with pairs of primers, derived from ten genes (CG17772, CG17771, CG13807, CG5714, CG13806, CG13805, CG5717, CG13804, CG13803, CG13802) occurring between the right breakpoints of the two deletions according to the BDGP (Berkeley *Drosophila* Genome Project; Fig. 1). CG5714 was identified as *ecd* by genetic rescue of the *ecd*$^{-}$ mutants. Genomic DNA from embryos or larvae homozygous for each of the *ecd* alleles was amplified with primers flanking the CG5714 gene: 5′-GGTACGAAGGAGGCGGAGGG-3′ and 5′-GATGAGCAAGATTCCAGGCAGCA-3′. PCR products from three independent reactions were sequenced using the BigDye Terminator Kit (Perkin Elmer), using these and additional internal primers to cover the entire *ecd* gene in both directions.

Transformation rescue of *ecd* mutants

Five genomic fragments containing the *ecd* candidate genes were obtained by restriction of the BACR22J16 clone (BDGP) and placed into the pCaSpeR-2 P-element vector (Thummel and Pirrotta, 1992). Clones E5, H13, B2, B13 and S4 (Fig. 1) were used for P-element-mediated germline transformation (Spradling and Rubin, 1982). *ecd*^{2}*/TM6B* and *ecd*^{g24}*/TM6B* females carrying the rescue construct *P[w*$^{+}$*, RC]* on the second chromosome: *w; P[w*$^{+}$*, RC]*; *ru ecd*^{2} *st e/TM6B* or *w; P[w*$^{+}$*, RC]*; *ve R ecd*^{g24}*/TM6B* were mated with males heterozygous for one of the *ecd* alleles (*ecd*^{1}, *ecd*^{2}, *ecd*$^{l(3)23}$, *ecd*^{g24} or *Df(3L)R+R2*) over *TM6B* to test for genetic rescue of *ecd*.

Lethal phase determination

Each *ecd* allele was crossed with all other *ecd* alleles and with the *Df(3L)R+R2* deficiency. All lines were balanced with *TM3, P[w*$^{+}$*, act-GFP]*. The flies were allowed to lay eggs on apple juice plates, supplemented with baker's yeast paste at 25°C, or at 29°C in the case of *ecd*^{1} crosses. Eggs were collected in two-hour periods, and embryos or larvae were identified as *ecd* homozygotes by the absence of the GFP-marked balancer.

Hormone feeding and titer determination

For the non-conditional *ecd*^{2} and *ecd*$^{l(3)23}$ mutants, 200 early-second instar larvae of each genotype were placed in vials with a sucrose-yeast medium containing 20-hydroxyecdysone (20E) at concentrations of 1 mg/ml (Garen et al., 1977; Freeman et al., 1999), 250 μg/ml, 50 μg/ml or zero, and animals progressing to the second molt or beyond were counted. The temperature-sensitive *ecd*^{1} mutants were tested for puparium formation as third instar larvae on the same media at 29°C. In all cases the homozygous *ecd* mutants were compared with their rescued counterparts carrying the S4 construct. Radioimmunoassay of total ecdysteroids was performed in whole-body homogenates as described (Jindra et al., 1994).

Rescue with ectopic Ecd expression

A full-length *ecd* cDNA (GH14368; BDGP) was subcloned into the pUAST P-element vector (Brand and Perrimon, 1993). Transgenic flies carrying the *UAS-ecd* construct in the *ecd*^{2} mutant background were crossed with *ecd*^{2} lines carrying transgenic Gal4 drivers to produce *UAS-ecd/Gal4; ecd*^{2}*/ecd*^{2}. Six drivers were tested for the ability to rescue the *ecd*^{2} lethal phenotype: *act-Gal4* (from Dr B. Edgar), *ptc-Gal4* (Bloomington stock #2017), *sev-Gal4* (from Dr P. Vilmos), *en-Gal4* (from Dr Y. Hiromi), *Aug21* and *Feb36* (Siegmund and Korge, 2001; Andrews et al., 2002). All lines were balanced with *TM3, Ser, P[w*$^{+}$*, act-GFP]*, so that *ecd*^{2} homozygotes could be identified at all developmental stages.

Generation of somatic and germline *ecd*$^{-}$ clones

Mutant clones deficient for either Ecd or MBF1 (control) proteins were generated by mitotic recombination using the FLP-FRT technique as described (Xu and Rubin, 1993; Theodosiou and Xu,

1998; Chou and Perrimon, 1996). To induce clones in the developing imaginal discs, *w, hs-FLP; P[w+, ub-GFP]61F FRT 80B* females were mated with *w; ru ecd2 FRT 80B/ TM3, P[w+, act-GFP]* or with *y w; mbf1 FRT 80B* males. Their progeny were heat-shocked as larvae for one hour at 38°C, 24-36 hours after egg laying; adult females were heat-shocked for 3 hours at 37°C to generate mutant clones in the ovarian follicle cells. To obtain *ecd*-null germline clones, females *w, hs-FLP; ru ecd2 FRT3L-2A/TM6B* were mated with *w; P[w+; ovoD1]3L-2X48 FRT3L-2A/TM3* males. Before reaching the second-to-third instar transition, the progeny was heat-shocked twice for 2 hours at 38°C (Theodosiou and Xu, 1998). Emerged *w, hs-FLP/w; ru ecd2 FRT3L-2A/P[w+; ovoD1]3L-2X48 FRT3L-2A* females were mated, examined for egg laying, and sacrificed for immunostaining of their ovaries 3-10 days later. Alternatively, germline clones were induced by heat shock for 1 hour at 38°C in adult females, and were analyzed 3-7 days later.

RNA hybridization

Poly(A)+ RNA was isolated using the QuickPrep mRNA Purification Kit (Amersham) and *ecd* and *mbf1* transcripts were detected on northern blots with full-length cDNA probes as described (Uhlirova et al., 2002). The same *ecd* probe, and its sense version (for control), was used for in situ hybridization of adult ovaries (Tautz and Pfeifle, 1989; Buszczak et al., 1999); detection was with anti-DIG alkaline phosphatase and the CBIP/NBT substrate (Roche).

ecd-lacZ expression

An *ecd-lacZ* reporter was constructed by cloning a 1.25 kb *ecd* upstream genomic region into the pCaSpeR-AUG-βgal vector (Thummel et al., 1988). The same regulatory sequence in the S4 construct was sufficient for the rescue of *ecd*-null mutants. The *ecd-lac*Z activity was detected in transgenic animals using a standard X-gal staining procedure.

Ecd antibodies, immunoblot and tissue staining

The central portion of Ecd (amino acids 270-429) was expressed from pET28a (Novagen) as a hexahistidine fusion protein in the BL21-CodonPlus (Stratagene) *E. coli* strain. The protein was affinity-purified on a Ni-NTA agarose column (Qiagen) under denaturing conditions, then partially re-natured by dialysis and used for rabbit immunization. The collected antiserum was affinity-purified using the entire Ecd protein, produced by the yeast EasySelect *Pichia* Expression Kit (Invitrogen) and immobilized on the AminoLink Plus Coupling Gel (Pierce). For western blots, embryos or larvae were homogenized in a denaturing sodium dodecylsulphate (SDS) buffer, and total protein (ca. 10 μg per lane) was analyzed by 10% SDS-PAGE. Blots were probed with the purified anti-Ecd antibody, diluted 1:5000. Detection was with a goat HRP-conjugated anti-rabbit antibody (1:4000) and a chemiluminescent substrate. Whole-mount immunostaining of larvae and adult gonads was performed according to standard procedures, with antibodies diluted as follows: anti-Ecd, 1:1000; anti-MBF1, 1:10,000 (Liu et al., 2003); anti-Orb (4H8 DSHB), 1:30 (Lantz et al., 1994); and anti-FasIII (7G10 DSHB), 1:30 (Patel et al., 1987). Secondary antibodies conjugated with Alexa Fluor 488, Texas-Red (Molecular Probes) and Cy3 (Amersham) were used at a dilution of 1:1000. Images were captured on Axioplan 100 and confocal LSM410 inverted laser scanning microscopes (Zeiss).

Results

Identification of the *ecdysoneless* gene

Genetic mapping placed *ecd* among 10 genes predicted by the Berkeley *Drosophila* Genome Project to be within region 62D. Four partially overlapping genomic fragments harboring subsets of these 10 genes (Fig. 1) were used for germline transformation. All three obtained transgenic lines carrying

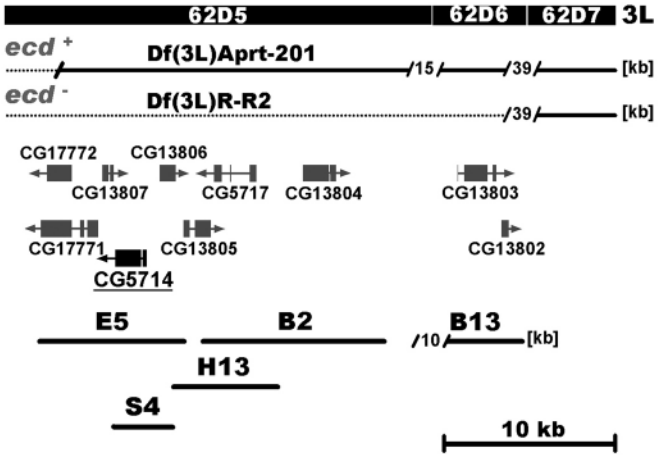

Fig. 1. Map of the *ecd* locus. The interval of *ecd* is delimited by the right breakpoints of deletions *Df(3L)Aprt201* and *Df(3L)R+R2* within the cytological region 62D5-D7 (top). Ten predicted genes (BDGP) located in this interval are shown. The filled boxes represent coding regions and the lines between them denote introns, arrows indicate the orientation of each gene. Five genomic fragments used for the preparation of rescue constructs are indicated by the black lines. CG5714 (underlined), present in the rescuing fragments E5 and S4, is the *ecd* gene.

the E5 genomic fragment rescued the otherwise lethal *ecd* genotypes: *ecd2/ecd2*, *ecd2/ecdl(3)23*, *ecd2/ecdg24*, *ecd2/Df(3L)R+R2*, *ecd1/ecd2* (29°C) and *ecd1/ecdg24* (29°C) to adulthood. A shorter construct S4, containing only the CG5714 gene (Fig. 1), rescued the *ecd* mutants to the same extent as E5. In all cases, a single transgenic copy of the CG5714 gene was sufficient for the complete rescue. These results clearly identify CG5714 as *ecdysoneless*.

The sequence of the deduced Ecd protein reveals a broad evolutionary conservation. Putative Ecd orthologs have been found in the mosquito *Anopheles gambiae* (43% overall amino acid identity), humans and mouse (31%), zebrafish (30%), *Arabidopsis thaliana* (26%) and the fission yeast *Schizosaccharomyces pombe* (21% identity). The human Ecd ortholog, known as Suppressor of GCR2 (SGT1), is expressed in a wide range of human organs (Sato et al., 1999) and functionally rescues a mutation of GCR2, a transcriptional regulator of glycolytic enzyme genes in the fission yeast (Deminoff and Santangelo, 2001). However, GCR2 is not homologous to SGT1 and thus the normal role of SGT1 in humans is unknown. Interestingly, although several highly conserved motifs are evident among the aligned orthologs (Fig. 2), none of these correspond to any known functional domain. There is a putative ATP/GTP-binding motif (P-loop) near the C terminus of the *Drosophila* and *Anopheles* orthologs, as recognized by the PROSITE database (Fig. 2).

Molecular basis of *ecd* mutations

To determine the character of mutations in aberrant *ecd* alleles, we have sequenced the relevant genomic region from *ecd* mutants. The temperature sensitive, EMS-induced allele *ecd1* contains a substitution of the conserved proline 656 to serine (Fig. 2), resulting from a C to T transition. All the other examined alleles: *ecd2*, and the two previously undescribed alleles *ecdg24* and *ecdl(3)23*, produce truncated Ecd peptides

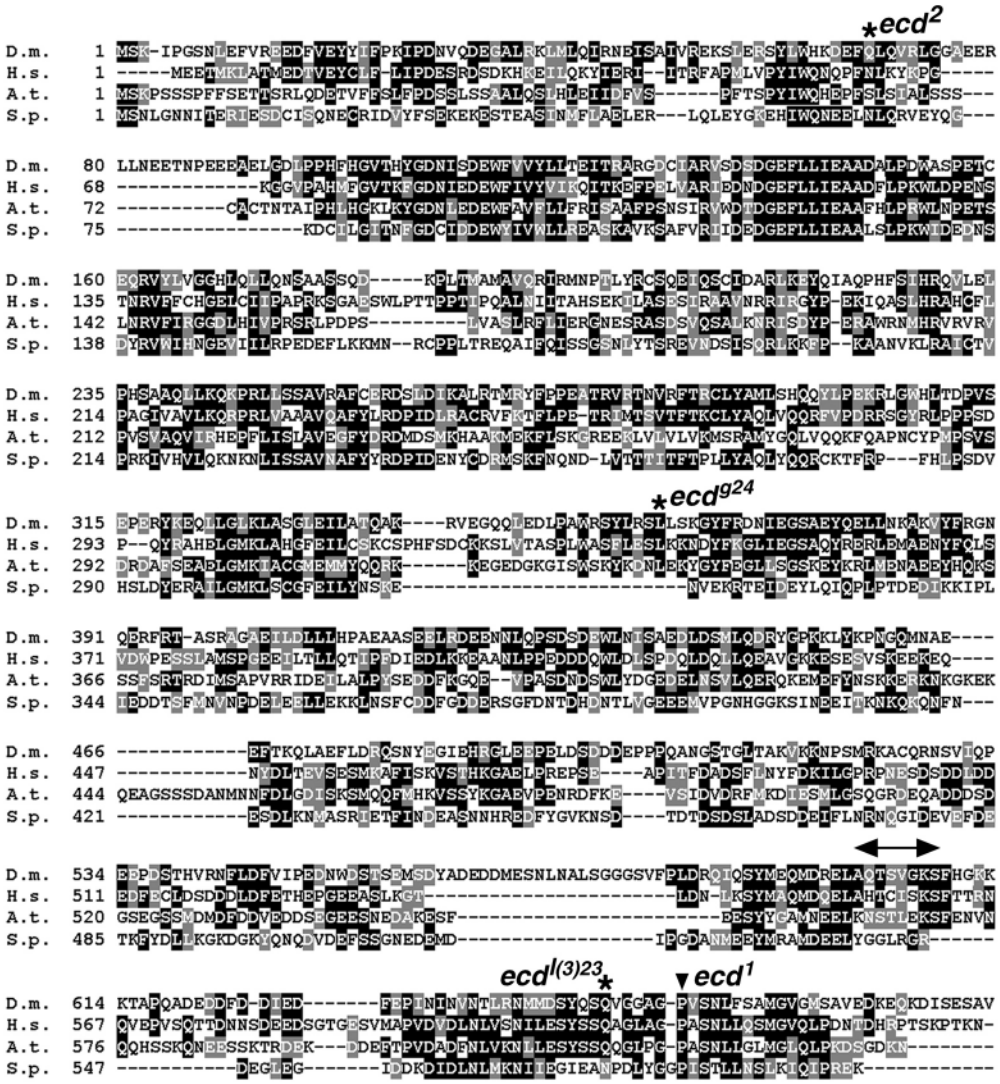

Fig. 2. Ecdysoneless is conserved throughout eukaryotes. Alignment of the *Drosophila* (Dm) Ecd protein (Swiss-Prot Q9W032) with human (Hs) SGT1 (O95905), and its closest relatives from *Arabidopsis thaliana* (At; Q9LSM5) and *Schizosaccharomyces pombe* (Sp; Q9US49), using Clustal W. Black shading indicates amino acid identity, and gray shading indicates similarity if present in two or more of the aligned sequences. The putative ATP/GTP-binding site (P-loop) is marked by the horizontal arrow. Asterisks indicate the positions of the premature termination codons in alleles ecd^2, ecd^{g24} and $ecd^{l(3)23}$. The arrowhead indicates the conserved proline 656, which is mutated to serine in ecd^1.

(Fig. 3A). The ecd^2 allele contains a C to T transition that converts Q_{67} to a stop codon. In the γ-ray induced ecd^{g24}, a four-base-pair deletion causes a frameshift of four amino acids followed by a stop codon. In $ecd^{l(3)23}$, the premature termination codon results from a C to T transition at Q_{650}. The extent of the presumed Ecd protein truncations suggests that ecd^2, at least, is a null allele. In agreement with the described mutations, a specific antibody raised against a central portion of the Ecd protein detected a wild-type sized band on western blots from third instar ecd^1 larvae (29°C), but not from ecd^2, ecd^{g24} or $ecd^{l(3)23}$ homozygotes approaching their lethal phases (Fig. 3B; data not shown for ecd^{g24}). A truncated Ecd product was found in $ecd^{l(3)23}$ homozygotes (Fig. 3B).

The lethal stage of the *ecd* mutants was examined to establish whether the structural character of the mutations corresponded to their phenotypic effects (Table 1). The single proline-to-serine substitution in ecd^1 is consistent with previous (Henrich et al., 1993; Sliter, 1989), and with our own, indications that the mutant gene product retains a residual function. Although most ecd^1 homozygotes completed their second molt at 29°C, the majority of the $ecd^1/Df(3L)R+R2$ hemizygotes, and ecd^1/ecd^2 and $ecd^1/ecd^{l(3)23}$ heteroallelic mutants, died during the second molt, displaying typical molting defects such as double mouth hooks (Fig. 4A). Among the non-conditional mutants, ecd^{g24} homozygotes were the most severely affected (Table 1), and $ecd^{g24}/Df(3L)R+R2$ larvae arrested during the first molt with unshed cuticles and double mouth hooks (Fig. 4B,C). This early lethality could be

Table 1. Lethal phases of *ecd* mutants

Genotype	Major lethal stages (% *n*)	Number of larvae (*n*)
ecd^1/ecd^1 *	L2 and early-L3 (12); mid-L3 (78)	200
$ecd^1/Df(3L)R+R2$	L2-L3 molt (75); L3 (25)	80
ecd^1/ecd^2	L2-L3 molt (65); L3 (35)	120
$ecd^1/ecd^{l(3)23}$	L2-L3 molt (83); L3 (17)	125
ecd^2/ecd^2	Prolonged L2	500
$ecd^2/Df(3L)R+R2$	Prolonged L2	60
$ecd^2/ecd^{l(3)23}$	Prolonged L2	75
$ecd^{l(3)23}/ecd^{l(3)23}$	Prolonged L2	200
$ecd^{l(3)23}/Df(3L)R+R2$	Prolonged L2	90
ecd^{g24}/ecd^{g24}	L1 (100)	100
$ecd^{g24}/Df(3L)R+R2$	L1 (43); L1-L2 molt (57)	120

*All genotypes containing ecd^1 were tested at 29°C.

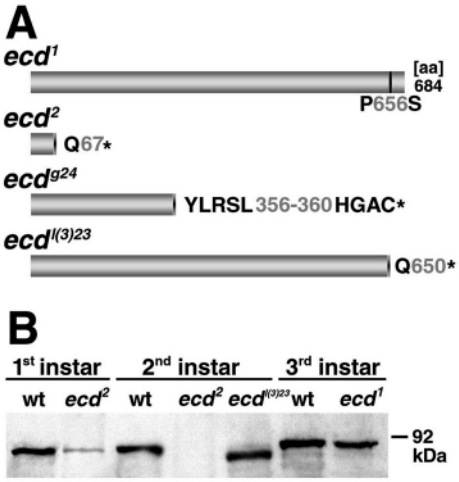

Fig. 3. Structure and expression of mutant Ecd forms. (A) Schematic representation of mutant versions of the Ecd protein in the four studied *ecd* alleles. Numbers are amino acid positions; asterisks denote stop codons. A 4-bp deletion in the γ-ray induced mutant ecd^{g24} changes the reading frame, resulting in the indicated amino acid substitutions and a stop codon. (B) Western blot of the wild-type and mutant Ecd forms. A low amount of maternal Ecd is still visible in the first instar, but not in the second instar homozygous ecd^{2} larvae. The truncated Ecd version is detected by the anti-Ecd antibody in second instar $ecd^{l(3)23}$ larvae.

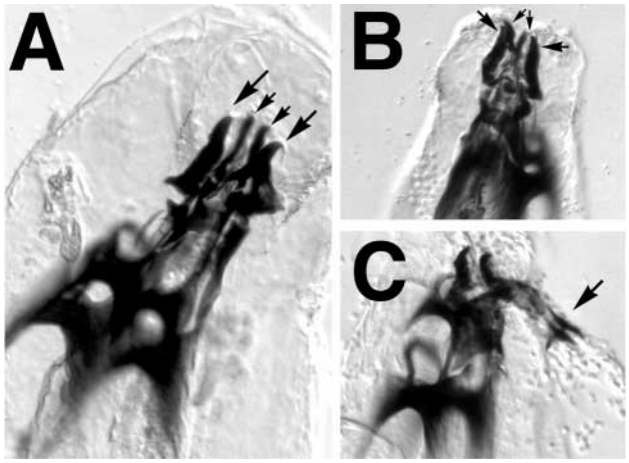

Fig. 4. Molting defects of *ecd* mutants. (A) Presence of the second instar (smaller arrows) and third instar (larger arrows) mouth hooks in an ecd^{1}/ecd^{2} heterozygous larva kept at 29°C. (B) Two pairs of mouth hooks in an $ecd^{g24}/Df(3L)R+R2$ hemizygote that died during the first molt. The first instar mouth hooks are indicated by small arrows and the second instar ones are indicated by larger arrows. (C) First instar cuticle attached through the first instar mouth hooks (arrow) to the new cuticle in an ecd^{g24} hemizygote.

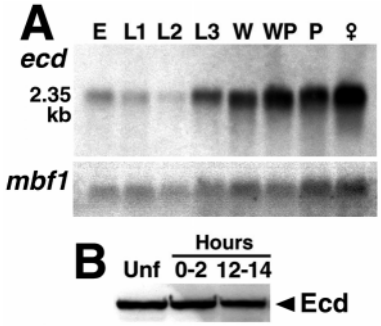

Fig. 5. Developmental expression of *ecd*. (A) Northern blot analysis showing expression of an *ecd* mRNA in embryos (E), in first (L1), second (L2) and third (L3) instar larvae, in wandering larvae (W), in white puparia (WP), in pupae (P) and in egg-laying females (add female symbol). Bottom panel shows a control re-hybridization of the blot with the constitutively expressed gene *mbf1*. (B) Western blot with the anti-Ecd antibody detects Ecd in unfertilized eggs from virgin mothers (Unf) and embryos 0-2 hours and 12-24 hours after egg laying.

in part caused by the dominant *Roughened (R)* mutation, or by another unknown mutation, on the ecd^{g24}-bearing chromosome, as animals lacking most or all of the Ecd protein in ecd^{2} homozygous or heteroallelic combinations arrested during the second instar. The new $ecd^{l(3)23}$ mutation was as severe as ecd^{2} (Table 1). These results suggest that ecd^{2}, ecd^{g24} and $ecd^{l(3)23}$ likewise represent *ecd*-null alleles that completely prevent development beyond the second instar.

ecd is expressed in steroidogenic as well as non-steroidogenic tissues

Northern blot analysis of whole animals showed a single *ecd* transcript, present throughout development (Fig. 5A). The mRNA was more abundant towards the end of the final larval instar and during metamorphosis; the strongest expression was observed in mature, egg-laying females. In situ hybridization showed that this increase probably resulted from strong *ecd* expression in the ovarian nurse cells (Fig. 6M). The continuous *ecd* expression was confirmed at the protein level using a specific antibody that detected Ecd from early embryogenesis to adulthood (Fig. 5B, Fig. 3B, and data not shown). Ecd was found in unfertilized eggs, showing maternal deposition of the protein (Fig. 5B). A western blot of early larvae homozygous for the ecd^{2} null allele revealed that low levels of the maternal Ecd protein persisted into the first larval instar (Fig. 3B).

A steroidogenic role of Ecd presumes its presence in the sites of ecdysone synthesis. Staining of late-third instar larvae revealed Ecd expression in the steroidogenic lateral lobes of the ring gland (Fig. 6A,B). However, ring glands of late embryos (not shown), and first or second instar larvae (Fig. 6C), did not show prominent staining. Also the rest of the body displayed only a diffuse signal without a restricted pattern. The ring gland temporal profile was corroborated by using a transgenic β-galactosidase reporter (*ecd-lacZ*), which was active only in the medial corpora allata region but not in the lateral steroidogenic gland of second instar larvae (Fig. 6D). This construct strongly labeled the whole ring gland in late third instar (Fig. 6E). Except for the medial ring gland, not stained with the antibody (Fig. 6A), the *lacZ* reporter probably reflected true *ecd* expression, because it was driven by an *ecd* upstream genomic region that is sufficient for the rescue of *ecd* mutants. Besides the ring gland, specific Ecd expression was found in the nervous system (Fig. 6G), in the imaginal discs (Fig. 6H,I) and in developing gonads of third instar larvae (Fig. 6J,K). In all cases the Ecd protein predominantly resided in the cytoplasm.

During metamorphosis the lateral ring gland degenerates. Other organs, such as ovaries, serve as sources of adult ecdysone. In adult ovaries, Ecd protein was expressed in both the somatic follicle cells and the germline nurse cells throughout oogenesis (Fig. 6L). The signal was stronger in the nurse cells of egg chambers staged 8-10, probably because of the deposition of the Ecd protein, as well as mRNA (Fig. 6M) into the oocyte at this time. High levels of Ecd were detected in the apical part of adult testes, where the somatic and germline stem cells are localized and where spermatogenesis begins (Fig. 6N). In summary, Ecd expression was detected in the primary steroidogenic organs – the larval lateral ring gland and the adult ovaries – as well as in the non-steroidogenic central nervous system and imaginal discs.

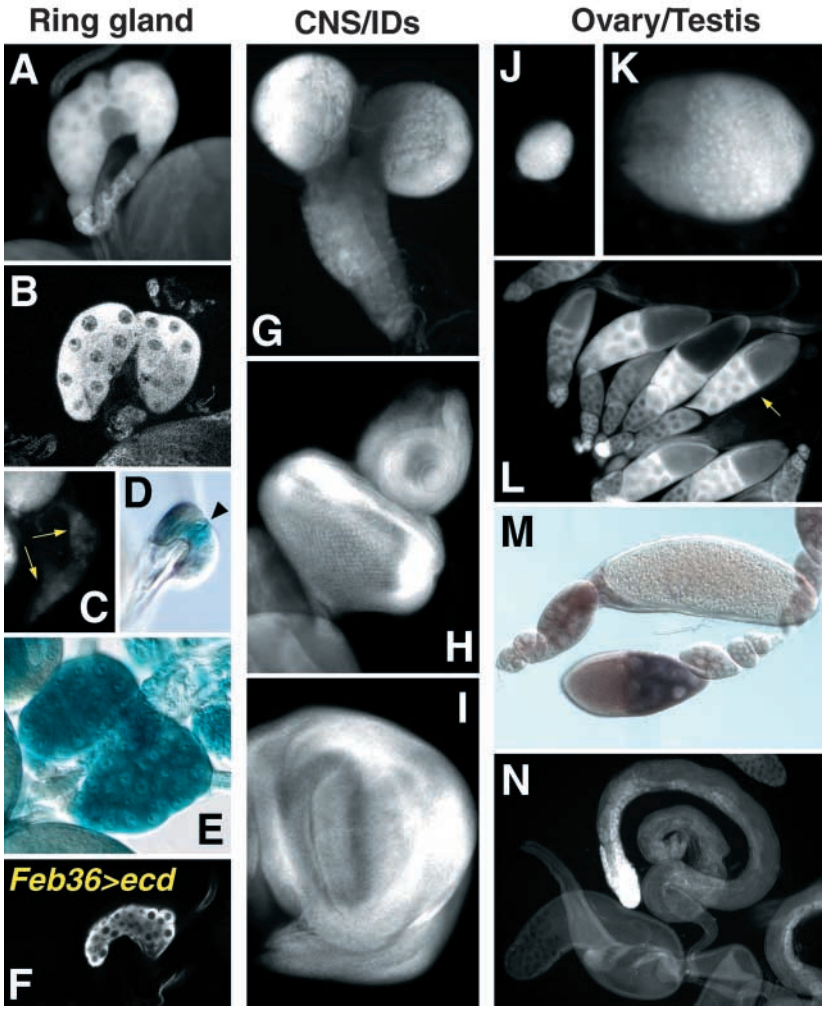

Fig. 6. *ecd* is expressed in specific steroidogenic, non-steroidogenic and reproductive organs. (A-F) The anti-Ecd antibody strongly stains the lateral lobes of a third instar ring gland (A); a confocal image (B) shows that Ecd is in the cytoplasm. Low signal is seen in the ring gland during the second instar (C, arrows). Activity of an *ecd-lacZ* reporter in the ring gland of second instar (D) and late third instar (E) larvae is visualized with X-gal staining. Arrowhead in D marks the medial corpora allata region. (F) Anti-Ecd antibody staining shows Ecd expression targeted to the lateral ring gland of a *Feb36-Gal4; ecd^2, P[UAS-ecd]* second instar larva. (G-I) A third instar CNS shows moderate Ecd levels (G) compared with the lateral ring gland (A). Ecd is abundant in the eye-antennal (H) and wing (I) imaginal discs. (J-N) The anti-Ecd antibody shows strong expression in both the ovary (J) and the testis (K) of third instar larvae. High levels of Ecd occur in the nurse cells of stage 10 egg chambers (L, arrow). The same stage nurse cells also accumulate an *ecd* transcript, as shown by in situ hybridization (M). Ecd is present primarily on the apical end of the adult testis (N). Except for F, staining was performed on *white^{1118}* animals.

Hormone feeding or *ecd* expression in the ring gland cannot rescue non-conditional *ecd* mutants

The presence of Ecd in the late-third instar ring gland is consistent with the steroid deficiency for which *ecd^1* was originally identified. The ability to induce puparium formation by feeding the non-pupariating *ecd^1* larvae at 29°C with 20-hydroxyecdysone (20E) (Garen at al., 1997; Redfern and Bownes, 1983; Berreur et al., 1984), suggested that low steroid levels might be the primary cause of arrest at this stage. To test whether the non-conditional *ecd* mutants could also be rescued by dietary hormone, we fed homozygous second instar *ecd^2* and *ecd$^{l(3)23}$* larvae with 20E. The feeding of *ecd^1* larvae at 29°C served as a positive control: 50 μg/ml and 250 μg/ml 20E doses induced pupariation in 26 out of 100, and in 36 out of 100, *ecd^1* homozygotes, respectively. By contrast, none of 600 *ecd^2*, or 250 *ecd$^{l(3)23}$*, larvae progressed beyond their lethal phase when fed 20E. These results strongly imply that ecdysone deficiency alone does not account for the second instar lethality of these mutants. In support of this view, the whole-body ecdysteroid content was not significantly different between *ecd^2/ecd^2* (0.61±0.13 pg/animal) and *ecd$^+$* (0.48±0.08 pg/animal) first instar larvae.

To address the problem of *ecd* requirement directly, we have targeted *ecd* expression to the steroidogenic part of the ring gland using transgenic *UAS-ecd* activated by a Gal4 driver, *Feb36* (Siegmund and Korge, 2001; Andrews et al., 2002). As was expected from the ability of exogenous 20E to rescue pupariation of *ecd^1* homozygotes at 29°C, Ecd expressed under *Feb36* allowed formation of defective puparia in around 25% *UAS-ecd, ecd^2/ecd^1* larvae upshifted to 29°C (*n*=60). The ectopic Ecd presence in the ring gland, evident during the second instar (Fig. 6F), should therefore restore the impaired hormone synthesis and at least postpone the arrest of *ecd*-null mutants, if disrupted ecdysone production was the sole cause of their death. However, the *Feb36*-driven Ecd was insufficient to advance *UAS-ecd, ecd^2/ecd^2* larvae even to the second molt. By contrast, the same *UAS-ecd* construct expressed under a ubiquitous *actin-Gal4* driver allowed *ecd^2* homozygotes to reach adulthood (Table 2).

The failure to rescue non-conditional *ecd* mutants with Ecd targeted to the ring gland, or by 20E feeding, correlates with the absence of Ecd from the ring gland before the third instar. Taken together, the data show that *ecd* is autonomously required in other organs before it is needed for ecdysone synthesis. To identify the

Table 2. Rescue of *ecd²* homozygotes by Gal4-targeted Ecd expression

Gal4 driver	Expression domain in larvae*	Rescue (% *n*)	Number of larvae (*n*)
actin/+	Ubiquitous	Adult (46)	80
Feb36/+	Lateral ring gland, salivary glands, fat body, malpighian tubules, tracheae, some midgut cells	None	200
Aug21/+	Medial ring gland, salivary glands, some midgut cells, malpighian tubules, tracheae	None	100
patched/+ (*ptc/+*)	Imaginal discs, salivary glands, epidermis, gut, nervous system, fat body	L3 (71)	130
ptc/ptc	As above	PP (12)	65
Feb36/ptc	As *Feb36* plus *patched* above	L3 (75)	100
sevenless/+	Eye imaginal discs	None	120
engrailed/+	Posterior imaginal discs, part of nervous system	None	180

*Expression patterns of individual drivers were verified using a *UAS-lacZ* reporter.

tissue-specific requirement, we have expressed Ecd using several other Gal4 drivers (Table 2). Ecd driven by the *patched* (*ptc*) promoter provided a partial rescue: a single copy of *ptc-Gal4* allowed *ecd²* homozygotes to molt to the third instar; two copies supported formation of defective but tanned prepupae.

Cell-autonomous function of *ecd* in imaginal discs

To examine whether *ecd* plays a cell-autonomous role during development of the adult, we have generated mitotic clones homozygous for the null allele *ecd²* using the FLP-FRT system. Mutant clones of a non-essential gene, *mbf1* (Liu et al., 2003), located as *ecd* on the 3L chromosome arm, served as a control. For both genes, wild-type sister clones and the heterozygous background were recognizable by the presence of ubiquitin-driven GFP and the *mini white⁺* gene markers, placed on the homologous chromosome. When induced early during the first larval instar, large *mbf1*$^{-/-}$ as well as *mbf1*$^{+/+}$ clones appeared in the adult compound eyes. By contrast, only *ecd*$^{+/+}$ clones were found with *ecd²* (Fig. 7A,D). The lack of *ecd²/ecd²* clones was confirmed by staining of imaginal discs, dissected from late third instar larvae: homozygous mutant clones were only found in *mbf1* but not in *ecd* somatic mosaics (Fig. 7B-F). No defects were observed in the adult eyes, legs, wings or thorax derived from the imaginal discs where *ecd²/ecd²* clones were induced. As imaginal disc cells normally proliferate throughout larval life (Madhavan and Schneiderman, 1977), we assume that the *ecd*$^{-/-}$ cells were replaced by their *ecd⁺* neighbors. The loss of Ecd, however, does not seem to be immediately cell-lethal, because small *ecd*$^{-/-}$ clones could be seen in eye-antennal imaginal discs when induced at the onset of the third instar (not shown).

Ecd is required for oogenesis

Ecd clearly plays a role in oogenesis, as the restrictive temperature prevents development of egg chambers beyond stage 8 in *ecd¹* flies (Audit-Lamour and Busson, 1981). To test whether Ecd is autonomously required in the somatic follicle cells, we induced homozygous *ecd²* and control *mbf1* clones in adult females. Ovaries with *ecd*$^{-/-}$ clones displayed defective egg chambers with extranumerary nurse cells, often double the normal 15 (Fig. 8A,B). Staining with an antibody against Orb, a protein that accumulates in the developing oocyte, confirmed that the aberrant egg chambers resulted from fusions of adjacent cysts, and not by overproliferation of the germline cells (Fig. 8C,D). Fasciclin III (FasIII), normally expressed by one pair of specific follicle cells at each pole of each egg chamber (Fig. 8A′), was detected only at the opposite ends of a fusion between two egg chambers (Fig. 8E). Defective egg chambers that had probably fused from several cysts early in their development showed multiple oocyte precursors (Fig. 8F), as well as FasIII-positive islands of cells (Fig. 8G). None of these defects occurred in ovaries containing large *mbf1* mutant clones (not shown). These results show that *ecd* is required in the follicle cells for normal oogenesis.

To test for a direct role of Ecd in oocyte development, we induced *ecd²/ecd²* germline clones using the FLP-FRT system with the *ovo^{D1}* dominant female sterile marker. When recombination was induced during the first larval instar, control *ovo^{D1}* females laid eggs, whereas females (*n*=50) carrying the *ecd²* mutation over *ovo^{D1}* did

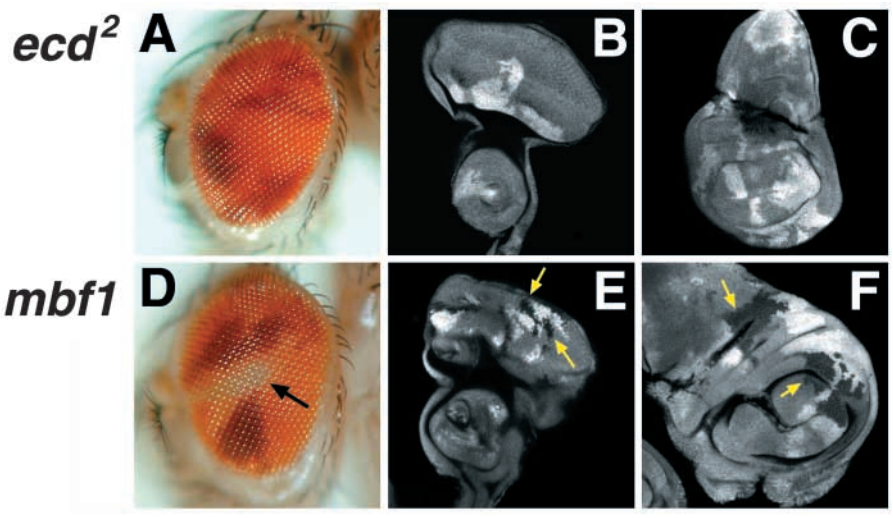

Fig. 7. Ecd-deficient cells do not survive in proliferating imaginal discs. *ecd²/ecd²* (top row) and *mbf1/mbf1* (bottom row) null mutant clones were induced during the first larval instar. Only the sister *ecd*$^{+/+}$ clones, marked by the intensive expression of *P[w⁺, ub-GFP]*, are found in the adult eye (A), eye-antennal discs (B) and wing discs (C), whereas *ecd⁻* cells are absent. Clones lacking *mbf1*, *P[w⁺, ub-GFP]* are maintained in all these imaginal tissues (D-F, arrows).

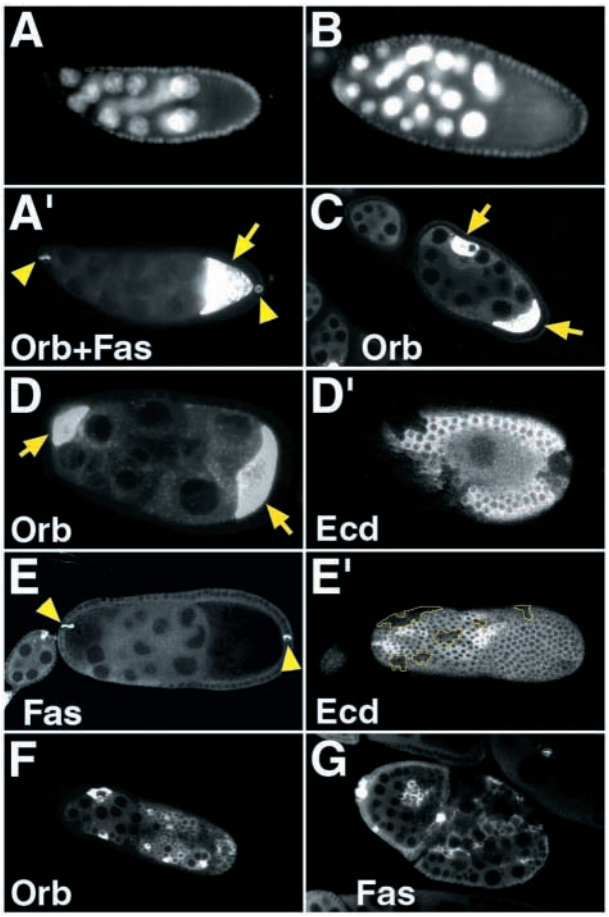

Fig. 8. Egg chambers carrying clones of *ecd*^{2}/*ecd*^{2} follicle cells fuse together. The normal number of 15 nurse cells in a control vitellogenic egg chamber (A) frequently doubles (B) when *ecd*$^{–}$ clones are induced in adult females. Presence of two (C,D) or more (F) oocytes confirms egg chamber fusions. Fasciclin III expression appears at the ends of a double egg chamber, but not at the fusion line (E). Multiple FasIII signals may result from a fusion of several cysts (G). (A,A′) Images show the same wild-type egg chamber. (D,D′,E,E′) Images show Orb, FasIII and Ecd proteins in the same egg chambers. Large clones of *ecd*$^{–}$ cells are clearly visible (D′) and smaller clones are emphasized with yellow lines (E′). Except for DNA staining with DAPI (A,B), all images are confocal sections. Arrows indicate Orb-positive oocytes; arrowheads indicate FasIII-positive polar follicle cells. Anterior is to the left.

not. Their ovaries contained clonal egg chambers that did not stain with the anti-Ecd antibody (Fig. 9A,B) and that arrested prior to vitellogenesis. When recombination was induced in adult females, some of them laid a few eggs (on average 1 per female; *n*=70) 5-6 days later. Ovaries dissected 3 days after the induction contained mosaic egg chambers, in which some nurse cells lacked the Ecd protein, whereas others strongly stained with anti-Ecd antibody (Fig. 9C,D). Interestingly, only these *ecd*$^{+}$/*ecd*$^{–}$ egg chambers progressed to vitellogenic stages, whereas those entirely devoid of Ecd arrested very early, showing degeneration of the nurse cells. Apparently the *ecd*$^{+}$, *ovo*^{D1} nurse cells and their adjacent *ecd*$^{–}$, *ovo*$^{+}$ sisters mutually rescued each other, thus allowing further development of the oocyte.

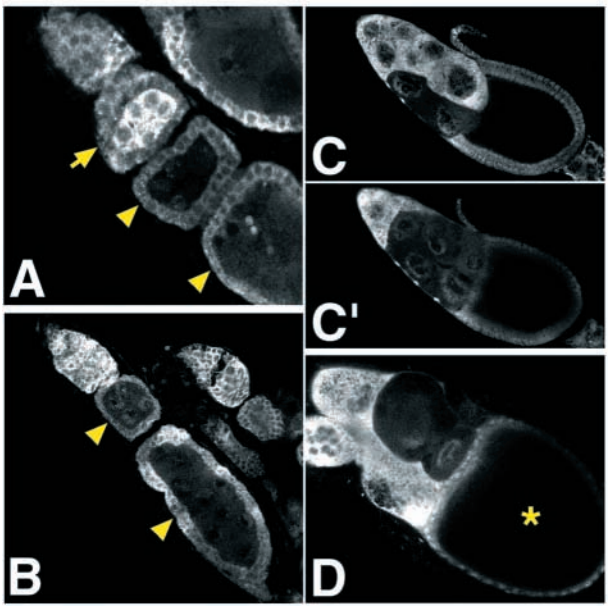

Fig. 9. Loss of *ecd* in the germline causes previtellogenic arrest of egg chambers. (A,B) *ecd*^{2}/*ecd*^{2} germline clones (arrowheads) can be distinguished from an *ecd*$^{+/+}$ clone (arrow) by the lack of staining with the anti-Ecd antibody. All of these clones arrest because of the loss of Ecd, or because of the *ovo*^{D1} mutation. (C,D) Mosaic egg chambers containing both Ecd$^{–}$ and Ecd^{+} nurse cells were identified with the anti-Ecd antibody three days after *ecd*^{2} clones had been induced in adult females. Only such mosaic egg chambers formed vitellogenic oocytes (asterisk). (C,C′) Confocal sections through the same egg chamber.

Discussion

The temperature-sensitive *Drosophila* mutation *ecd*^{1} has been widely used as an ecdysone-deficient background for developmental studies despite uncertainty about its molecular identity and other possible roles of the *ecdysoneless* gene. The aim of this study is to show that *ecd* encodes a conserved protein, previously not connected with steroid biosynthesis or any other function, and to demonstrate that besides its known steroidogenic role, this protein is required in a cell-autonomous manner independently of the blood-circulating hormone.

We have mapped molecular defects in the original *ecd*^{1} (Garen et al., 1977), in *ecd*^{2} (Sliter et al., 1989), and in two previously undescribed alleles, *ecd*$^{l(3)23}$ and *ecd*^{g24}. The point mutation found in *ecd*^{1} is consistent with its hypomorphic nature (Henrich et al., 1993). It converts a proline residue, conserved in all Ecd orthologs identified so far, into serine. This substitution does not cause degradation of the Ecd protein (Fig. 3B), or its subcellular mislocalization in the ring gland at 29°C (not shown). The mutation maps near the C terminus (Fig. 2), which must harbor an important function because a short truncation in *ecd*$^{l(3)23}$ lacking this region is phenotypically as severe as the *ecd*^{2} mutation, removing almost the entire protein (Fig. 3A). Although the non-conditional *ecd*$^{–}$ mutants die as second instar larvae, temperature shifts of the *ecd*^{1} mutants suggest that Ecd is required during embryogenesis (Kozlova and Thummel, 2003). This early function may be executed by the maternally supplied Ecd protein, which is still detectable in first instar *ecd*$^{–}$

homozygotes (Fig. 3B). As the effects of *ecd*^{2}, *ecd*$^{l(3)23}$ and *ecd*^{g24} are not worsened in hemizygous combinations with an *ecd*$^{-}$ deficiency, all of these three mutations are likely to represent *ecd*-null alleles. A single transgenic *ecd*$^{+}$ copy rescues all *ecd*$^{-}$ mutants to adulthood, showing that the developmental and lethal defects seen in these mutants are fully attributable to the loss of *ecd* function.

Although the non-conditional *ecd*$^{-}$ mutants often die during the ecdysis to the second instar, displaying phenotypes that might imply defective ecdysone production (Fig. 4B,C), their lethality cannot be a direct consequence of low blood ecdysone for the following reasons. First, *ecd*$^{-}$ animals cannot be advanced to the second molt by 20E feeding, despite the fact that similar doses of 20E are sufficient (1) to avert second instar lethality in mutants for the steroidogenic enzyme Dare (Freeman et al., 1999) and (2) to induce pupariation in *ecd*^{1} larvae at 29°C. Second, as some of the *ecd*$^{-}$ animals die during the transition to the second instar, one would expect that their ecdysone titer would be lower from as early as the first instar. However, we have not found a reduction of ecdysone content in first instar homozygous *ecd*^{2} larvae. Third, although Ecd is abundant in the lateral ring gland during the third instar, no such expression is seen at earlier stages. By contrast, some other steroidogenic genes, such as *dib* and *sad*, are strongly expressed in the ring gland beginning at embryogenesis (Chavez et al., 2000; Warren et al., 2002). Finally, development of *ecd*^{2} homozygotes can be completely rescued with ubiquitous Ecd expression but not with Ecd targeted by the *Feb36-Gal4* driver to the ring gland and to some other organs (Andrews et al., 2002). As Ecd presence in the ring gland cannot postpone the death of *ecd*-null mutants, Ecd must be required prior to the initiation of the second molt in some other tissues. One could be the nervous system (Fig. 6G), because *patched*-driven Ecd promotes further development of the mutants.

A cell-autonomous effect was previously demonstrated for the *ecd*^{1} allele during differentiation of the thorax sensory bristles (Sliter, 1989). Unexpectedly, induction of *ecd*-null mitotic clones in the primordia of the adult thorax, the wing imaginal discs, did not produce any defective bristles. This was probably because no *ecd*$^{-}$ clones occurred in the adult epidermis. Based on the presence of twin *ecd*$^{+/+}$ clones in all imaginal discs and in the adult compound eye (Fig. 7), we conclude that the lost *ecd*$^{-}$ clones were replaced by proliferation of the surrounding *ecd*$^{+}$ cells. Redfern and Bownes (Redfern and Bownes, 1983) ascribed many of the defects seen in temperature-upshifted *ecd*^{1} mutants to autonomous cell lethality in the imaginal discs. However, we have detected small clones of *ecd*$^{-}$ cells in imaginal discs upon induction of recombination during early third larval instar, and *ecd*$^{-}$ clones also survived in the adult ovary. Thus, the loss of *ecd* is not generally cell lethal although it reduces the ability of the mutant cells to proliferate at the normal rate. Our mosaic analyses provide direct evidence for a cell-autonomous, ecdysone-independent function of *ecd*, which may underlie the previously described defects in adult morphogenesis.

Clones of *ecd*$^{-}$ somatic follicle cells caused profound defects, manifest as fusions of adjacent egg chambers and leading to duplications of the nurse cell set, in some cases with two vitellogenic oocytes present at the opposite poles (Fig. 8D). Similar polarity defects were caused by perturbing the Delta/Notch signaling that specifies the polar follicle cells (PFC), and by perturbing the JAK/STAT pathway through which these cells establish proper separation between egg chambers (Gonzalez-Reyes and St Johnston, 1998; Grammont and Irvine, 2001; McGregor et al., 2002; Torres et al., 2003). It remains to be tested whether the egg chamber fusions in *ecd* mosaic ovaries might result from a compromised signaling by the PFC. Follicle cells are thought to be the major site of ecdysone production in the ovary (Lagueux et al., 1977; Zhu et al., 1983). However, it is difficult to imagine that the relatively small *ecd*$^{-}$ clones could significantly reduce the ecdysone titer in the female. Therefore we conclude that, as in the case of imaginal discs, the effects of *ecd*^{2} on oogenesis are independent of free-circulating ecdysone.

Germline clones completely lacking *ecd* function arrest at pre-vitellogenic stages, probably earlier than egg chambers carrying the *ovo*^{D1} mutation, thus showing that *ecd* is autonomously required for oocyte maturation. This result is consistent with the phenotype of *ecd*^{1} mutant ovaries: *ecd*^{1} females become sterile after a few days at 29°C, with a majority of egg chambers at pre-vitellogenic stages (Audit-Lamour and Busson, 1981). Interestingly, the steroidogenic enzyme Dare, and the ecdysone response proteins EcR and E75, are similarly required in the nurse cells for egg maturation, as germline clones mutant for these genes arrest as pre-vitellogenic egg chambers as well (Buszczak et al., 1999). This led the authors to propose that ecdysone synthesis by the germline is necessary in an autocrine manner for the progression of oocytes to the vitellogenic stage. As normal *ecd* function is required for autonomous ecdysone production by the ovary (Garen et al., 1977), the pre-vitellogenic arrest of the *ecd*$^{-}$ germline clones is consistent with an autocrine germline function.

By inducing *ecd*^{2} mutant clones in adult females, we created mosaic egg chambers in which some nurse cells were null for *ecd*, whereas others carried the *ovo*^{D1} dominant mutation that unconditionally blocks oogenesis. Surprisingly, these mixed-genotype egg chambers continued to mature much beyond the phase of arrest caused by either the *ecd*^{2} or *ovo*^{D1} mutations acting alone (Fig. 9). This suggests a functional rescue among the cells within the egg chamber. As nurse cells are interconnected by ring canals, we speculate that the *ecd*$^{+}$ *ovo*^{D1} cells and their *ecd*$^{-}$, *ovo*$^{+}$ sisters exchanged materials that complemented them and consequently permitted oocyte development. In the light of the autocrine germline hypothesis (Buszczak et al., 1999), an intriguing possibility is that the product of the *ecd*$^{+}$ *ovo*^{D1} clones might be ecdysone.

Although the *ecdysoneless* gene encodes a protein with highly conserved regions, we have found no data that would describe the function of these regions and thus enlighten the mode of Ecd action. The only published report has implicated the human ortholog of Ecd, which compensates for the loss of an unrelated yeast protein GCR2 in transcriptional regulation (Deminoff and Santangelo, 2001). Our antibody detects Ecd predominantly in the cytoplasm, and thus does not directly support the possibility that Ecd acts at the level of transcription. We have initiated yeast two-hybrid studies to address the mechanism of Ecd action by identifying its protein partners. Until the exact function of Ecd is known, interpretations of results obtained with the ecdysone-deficient *ecd*^{1} mutants should consider its non-steroidogenic effects.

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